Identification and characterization of a novel splice variant of MuSK

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Abstract MuSK is a receptor tyrosine kinase that initiates the formation of neuromuscular junctions in response to agrin. Little is known about the ligand-induced activation and kinase-dependent signalling that leads to the clustering of acetylcholine receptors. The ectodomain of these molecule is composed of four Ig-like domains. We describe here the isolation of a novel MuSK splice variant that lacks the third Ig-like domain in its ectodomain. The corresponding RNA is the result of alternative splicing which eliminates two exons. There is 10 times less mRNA for this shorter form than for the long form of MuSK and both forms are regulated coordinately. They decrease strongly after birth and are elevated in denervated muscle. Gene transfer by muscle injection of MuSK DNA into individual muscle fibers demonstrates that kinase-induced acetylcholine receptor clustering caused by overexpression of the two kinases does not depend on the presence of the third Ig-like domain.

Key words: Muscle-specific tyrosine kinase; MuSK splice variant; Gene transfer

1. Introduction

Receptor tyrosine kinases at the cell surface are involved in the regulation of metabolic and signalling pathways and contribute to cell growth, differentiation, migration and apoptosis. They contain a cytoplasmic intrinsic kinase domain which is connected by a transmembrane segment to the ectodomain. The ectodomain contains one or several copies of immunoglobulin-like domains, EGF-like domains, cysteine-rich domains or other domains that form ligand binding or regulatory domains. Based on their structural characteristics the tyrosine kinase receptors can be classified into families [1]. The muscle-specific kinase, called MuSK [2], is unique in that it is specific to the skeletal muscle cell lineage and is part of a receptor complex that initiates neuromuscular junction formation in response to agrin [3]. In genetically engineered mice lacking MuSK, every aspect of synapse formation appears to be blocked, showing that this protein is necessary for postnatal life [4,5]. Agrin/MuSK-mediated acetylcholine receptor (AChR) clustering probably requires ectodomain regions of MuSK [6].

The ectodomain of MuSK contains three immunoglobulin (Ig)-like domains followed by a C6 box and a fourth Ig-like domain [2] also recognized recently as a frizzled cysteine-rich domain [7,8]. MuSK was isolated from human, rat and mouse muscles (nsk2) and several isoforms with minor differences resulting from alternative splicing have been described [2,9]. Using RT-PCR we isolated a novel MuSK cDNA, MuSK-ΔIgIII, in which the third Ig-like domain is missing. We show that MuSK-ΔIgIII transcripts resulting from alternative splicing and expression in muscle are regulated in a similar way to that observed for the longer, predominant MuSK form. Since ligand binding or interactions with regulatory proteins could differ between the two isoforms, we injected the corresponding DNA directly into muscle fibers to compare their AChR-clustering activity (Sander et al., submitted). Both MuSK isoforms induce AChR clusters upon overexpression independent of the presence or absence of the IgIII domain.

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Abbreviations: AChR, acetylcholine receptor; MuSK, muscle-specific kinase

2. Materials and methods

2.1. Cloning of MuSK cDNA

MuSK cDNA was isolated using RT-PCR (Titan Kit, Boehringer Mannheim, Germany). Total RNA was prepared from denervated rat hind leg muscles [10]. The sense primer, CTAGAATTCACCTTCTTCCTGAGCCTGG, contained an *Eco*RI site in the 5′ position of the ATG and the antisense primer, CTATCTAGACTCCTGGGTGTTGTTTGAGC, an *Xba*I site in the 3′ position to the stop codon. The primer sequences are based on the published sequence of the rat MuSK cDNA [2]. RT cDNA synthesis was performed at 50°C. The PCR amplification was done according to the supplier's protocol. The resulting RT-PCR product consisted of two fragments of around 2500 bp. Both products were subcloned into the pSP72 vector (Promega, Mannheim, Germany) and sequenced using the ABI Prism 377 DNA Sequencer (Applied Biosystems, Weiterstadt, Germany) and the ABI Prism BigDye Ready Reaction Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). Full length clones corresponded to the MuSK cDNA as published by Valenzuela et al. [2] and to the IgIII-lacking form, MuSK-ΔIgIII cDNA, as shown in Fig. 1.

PCR reactions were performed in a Perkin Elmer Cetus DNA Thermal Cycler 480 (Perkin Elmer, Ueberlingen, Germany).

2.2. Isolation of fragments of the MuSK/nsk2 gene from a 129 SVJ mouse genomic library

Gene fragments carrying the IgIII exons were isolated by screening a lambda FIX II mouse (129 SVJ) genomic library (Stratagene, Heidelberg, Germany). Hybond-N nylon filters (Amersham Buchler, Braunschweig, Germany) were lifted and hybridized with a PCR-synthesized 198 bp IgIII-specific probe corresponding to nucleotides 646–842 of the rat MuSK cDNA [2]. Hybridization was performed at high stringency (42°C for 16 h, 50% formamide, 0.5% SDS, 5×Denhardt's solution, 50 mM phosphate buffer pH 6.5, 200 μg/ml sonicated salmon sperm DNA). Filters were washed in 2×SSC, 0.1% SDS at 42°C for 10 min, then in 1×SSC, 0.1% SDS at 50°C for 10 min and finally in 0.5×SSC, 0.1% SDS at 50°C for 20 min. Six positive clones were obtained. One clone was digested with *Hin*dIII yielding two ca. 6000 bp fragments which were subcloned into pSP72 vector. Both fragments hybridized with the IgIII-specific probe and were further analyzed (see Fig. 2) employing the following sequencing primers to identify exon/intron borders: a1 (CTGCGTGCTCCTGAATCC), a2 (CAGCAGGAACAGTTCAGAGCC), a3 (GCTCAGTGTGTCCTGGGGTG), b1 (GGTCGGGATAGGAGGTGTTG), b2 (GTCTCGATCACCCATGTGTG), b3 (CCACAGAGCGACCCAGGGTCC).

2.3. Determination of the exon distance by long-PCR

PCR was performed with the primers a3 and b2 using the isolated lambda clone as a template that contains the IgIII exons. For the

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PCR reaction, two mixtures were prepared. One mixture contained 1 μl of each primer (20 μM) and 7 μl of dNTPs (350 μM) and 16 μl H_2O. A second mixture contained 5 μl reaction buffer, 3.5 μl Mg_2Cl_2 (1.75 μM), 1 μl Taq-Polymerase (5 U/μl), 3 μl lambda DNA (1 μg) and 12.5 μl H_2O. Both mixtures were stored on ice until starting the following PCR cycles: denaturation at 93°C for 2 min; the cycle 93°C (10 s), 65°C (30 s) and 68°C (10 min) was repeated 10 times. The cycle 93°C (10 s), 65°C (30 s) and 68°C (10 min) with an additional elongation time of 20 s of each cycle was repeated 15 times finally followed by an elongation time of 7 min.

A ca. 11 kb fragment was obtained and cloned in the pCRII TOPO vector (Invitrogen B.V., Leek, The Netherlands).

Sequencing with the primers a3 and b2 revealed that exon A and exon B with their 5′ and 3′ intron sequences were located on this PCR fragment.

2.4. MuSK transcript analysis in rat muscle

RT-PCR was started using 5 μg of total RNA from rat hind leg muscles, random hexamers as primers and the SuperScript II reverse transcriptase (Life Technologies GmbH, Eggenstein, Germany). Aliquots of this reaction mixture were then employed for the PCR amplification of MuSK cDNA sequences: for the analysis of total MuSK transcript levels, kinase-specific primers were used together with 1 μl of the cDNA reaction mixture (5′ sense primer CTCCTGGGTGTTGTTTGAGC and 3′ antisense primer CTGGTCCTCCACCCCTGTC). PCR conditions were as follows: denaturation at 95°C for 2 min; 95°C (1 min), 65°C (15 s), 72°C (7 min), extension at 72°C (2 s) of each cycle. In order to estimate the different transcript levels in normal (N) and denervated (D) muscle, PCR amplification was stopped after different cycle numbers. The PCR products were analyzed by agarose gel electrophoresis imaged with the Cybertech CS-1 camera (Cybertech, Berlin, Germany) and evaluated densitometrically with NIH Image 1.40 software. (Analysis was performed on a Macintosh 7300/200 computer using the public domain NIH Image program, written by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, USA, part number PB93-504868.)

As shown in Fig. 3A,B, with increasing cycle numbers (22, 24, 26, 28 cycles) one remained in the exponential phase of amplification.

The different transcript levels of MuSK and MuSK-ΔIgIII in N and D muscle or at different developmental stages, at embryonic days 17 and 19 (E17 and E19), and at postnatal day 1 (P1), were estimated in a similar way using a 5′ sense primer GCAAAAGGAAGACGCAGGAC and a 3′ antisense primer GGTCGGGATAGGAGGTGTTG, revealing that the two isoforms yielded two differently sized PCR products. PCR was performed and analyzed as described above. A MuSK-ΔIgIII-specific RT-PCR was performed as described above with a 'MuSK-ΔIgIII'- specific 5′ sense primer (GGAAGTGGAGGAATGGAGCAA) and a 3′ antisense primer (TGGACGGGAATGTTGTTATG).

2.5. MuSK cDNA injection into muscle fibers

Four to five week old rats were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL, USA) at a concentration of 50 mg/kg and DNA was injected as described previously [11] (Sander et al., submitted).

MuSK and MuSK-ΔIgIII cDNAs cloned into the expression vector pRK5 [12] used for injection were purified by Qiagen column chromatography (Qiagen, Hilden, Germany). The final DNA solutions of 100 ng/μl contained 90 mM KCl and 15 mg/ml Fast green FCF (Sigma, Deisenhofen, Germany).

3. Results

3.1. Cloning of a novel MuSK cDNA

Denervated rat muscle contains strongly elevated MuSK transcript levels [2]. Using total RNA extracted from denervated rat hind leg muscle and RT-PCR to isolate full length MuSK cDNA, we obtained two differently sized products indicating the presence of at least two different mRNAs (Fig. 1A). Both RT-PCR products were cloned and sequenced. The longer form corresponded to the sequence published for rat MuSK carrying A454 instead of the 8 amino acid insertion (Fig. 1B). The shorter isoform, on the other hand, had the 8 amino acid insertion replacing A454. In addition, the third Ig-like domain, IgIII, was completely absent after alternative splicing to yield MuSK-ΔIgIII (Fig. 1C). This shorter isoform, therefore, provided a tool to test the functional importance of the IgIII domain in MuSK.

3.2. The IgIII domain is located on two exons

In order to find out whether the MuSK-ΔIgIII isoform could result from alternative splicing we used a mouse 129 SVJ genomic library to isolate fragments that carry the exon sequences encoding the IgIII domain. Rat (MuSK) and mouse (nsk2) cDNA sequences show 93% similarity. With a hybridization probe from the rat MuSK cDNA including nucleotides 646–842, six phage colonies were isolated. Digestion and hybridization with the IgIII-specific probe resulted in two DNA fragments. Both fragments were subcloned into the pSP72 plasmid vector. With sequencing primers (a1, a2, a3, b1, b2, b3) exon sequences and exon borders were determined (Fig. 2). The nucleotide sequences of splice donor and accep-

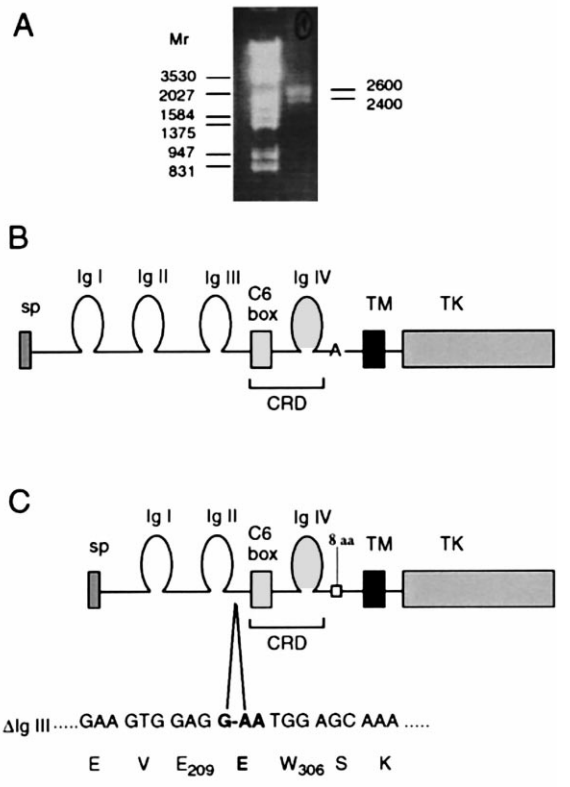

Fig. 1. Cloning of MuSK by RT-PCR. A: Total RNA from denervated rat muscle was used to generate full length cDNA of MuSK. PCR analysis of the cDNA fragments yields at least two products of different sizes shown on the right to the M_r standards (M_r sizes are indicated). The PCR products were cloned and sequenced. The apparent size of the PCR products is indicated on the right of the figure. B: The larger PCR product of A, shown schematically, yielded cDNA corresponding to the published MuSK cDNA [2] with an alanine residue at position A454 instead of an 8 amino acid insertion. C: The shorter PCR product of A contained only the first two Ig-like domains while the third domain, IgIII, was absent. The clone isolated carried instead of A454 the 8 amino acid insert as indicated schematically. sp, signal peptide; I–IV, Ig-like domains. C6 box and IgIV correspond to the frizzled cysteine-rich domain, CRD [7,8].

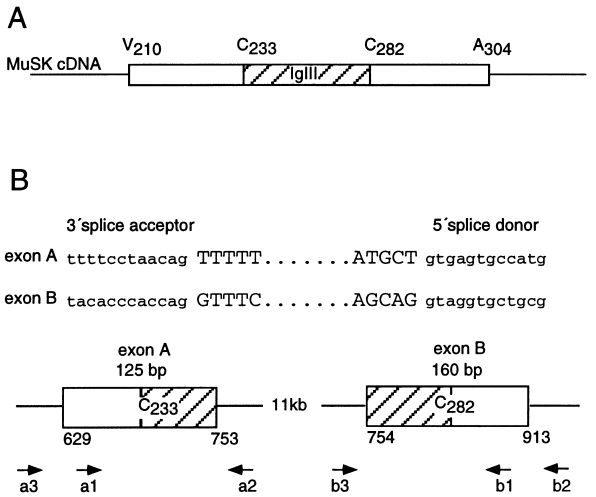

Fig. 2. Exon structure of the IgIII domain. Genomic DNA fragments of mouse 129 SVJ embryonic stem cells carrying the IgIII domain were isolated. The location of the exon sequences in the cDNA are shown schematically in A. Amino acid positions are identical in MuSK and nsk2 cDNA. The genomic exon/intron structure is outlined schematically in B. The amino-terminal cysteine of the IgIII domain with its 5′ flanking sequences are located on exon A while the carboxy-terminal cysteine and 3′ flanking sequences reside on exon B. The sequencing primers are indicated below and are described in Section 2.

tor sites strictly follow the GT-AG rule [13] and demonstrate that the IgIII domain is encoded by two separate exons. The two cysteines that form the intramolecular disulfide bond within the IgIII domain are separated by 48 amino acids.

3.3. MuSK isoform transcripts

MuSK transcripts were detected by Northern blot analysis shortly after birth (not shown) but levels decreased rapidly with postnatal development and they became restricted to the synaptic region [2,14]. Upon denervation, mRNA levels were strongly increased and thus appear to be regulated in a similar way to the activity-sensitive AChR subunit genes [14]. We used total RNA isolated from rat muscle at different developmental stages and from adult muscle as well as surgically denervated muscle for RT-PCR analysis. To estimate changes of transcript levels during development and to compare the relative amounts of MuSK and MuSK-ΔIgIII transcripts, increasing numbers of PCR cycles were run in the range of the exponential increase of PCR products. Fig. 3A,B shows the PCR analysis of total MuSK in adult normal and denervated muscle confirming the strong increase in MuSK mRNA following surgical denervation. Using primers that yield differently sized PCR products corresponding to MuSK and MuSK-ΔIgIII isoforms reveals that in denervated muscle the MuSK-ΔIgIII transcript levels are about 10 times less abundant than the MuSK transcripts (Fig. 3C). At prenatal stages and after birth, transcript levels of both isoforms were elevated when compared to the levels found in adult innervated muscle and the ratio of the two isoforms was apparently constant. The finding that MuSK-ΔIgIII transcript levels were elevated in embryonic and early postnatal muscle and upregulated in denervated muscle indicates that both MuSK isoforms are regulated similarly.

With a 'ΔIgIII specific'-primer pair (see Section 2) the strong denervation-induced transcript increase of MuSK-ΔIgIII was confirmed (not shown).

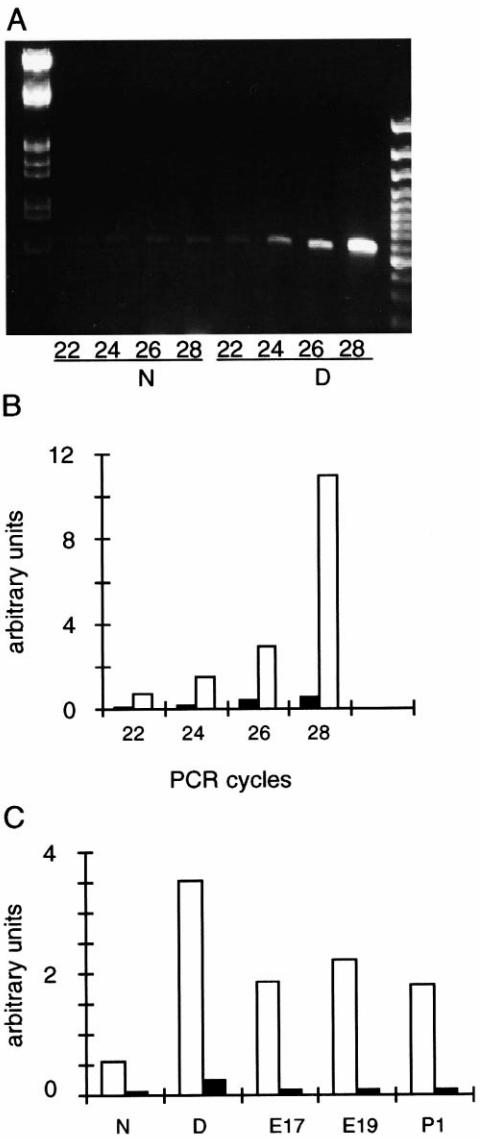

Fig. 3. MuSK transcript levels in rat muscle. Total RNA was extracted from rat hind leg muscle and analyzed by RT-PCR as described in Section 2. A: Total MuSK transcripts in adult innervated and denervated muscle amplified by kinase-specific primer pairs as described in Section 2. After 22–28 PCR cycles, 5 μl of the PCR products were applied to estimate MuSK transcript concentrations. N, PCR products from total RNA from normal, innervated muscle; D, PCR products from RNA from 5 day denervated muscle. Left and right lanes, M_r size standards. B: Densitometric evalution of lanes in A reveals 10-fold higher MuSK transcript levels in surgically denervated muscle. Normal muscle, black bars; denervated muscle, white bars. C: PCR products using primer pairs that reveal the different IgIII-containing and IgIII-lacking transcript levels. Total RNA was extracted from N, normal innervated muscle, D, denervated muscle, E17 and E19, from embryos at days 17 and 19, P1, from animals at postnatal day 1. MuSK, white bars; MuSK-ΔIgIII, black bars.

3.4. MuSK overexpression in muscle induces AChR clusters

We have developed an efficient DNA injection method which allows gene transfer into distinct muscle fibers of living animals [11]. MuSK cDNA cloned into the expression vector

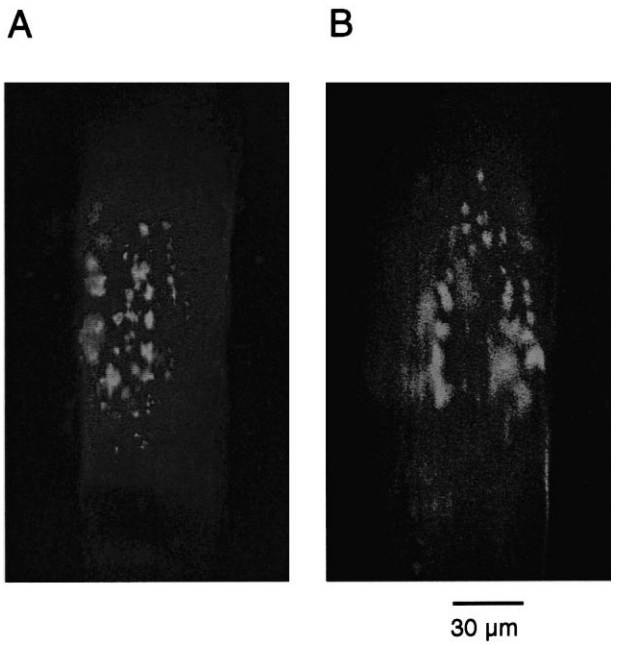

Fig. 4. Injection of MuSK into individual innervated muscle fibers induces AChR clusters at ectopic sites. MuSK (A) and MuSK-ΔIg-III (B) cDNA in pRK5 expression vector were injected as described in Section 2. In both cases AChR clusters were induced by the transgene-expressing muscle fiber as visualized with rhodamine-labelled α-bungarotoxin.

pRK5 induced, upon injection, the appearance of characteristic AChR clusters on the transgene expressing muscle fiber probably due to overexpression and the resulting autophosphorylation (Sander et al., in preparation). Since Ig-like domains could be part of a regulatory or binding domain interacting with synaptic components, we tested whether MuSK-ΔIgIII can still be activated to induce AChR clusters. The MuSK-induced AChR clusters were indistinguishable suggesting that autophosphorylation and AChR cluster-inducing activity do not depend on the presence of the third Ig-like domain (Fig. 4).

4. Discussion

MuSK shares homology with a muscle-specific tyrosine kinase cloned from the electric organ of the electric ray *Torpedo californica* [15] both having four Ig-like domains and a C6 box. The RTK-like proteins Ror1 and Ror2 [16] have only three Ig-like domains but, like the *Torpedo* enzyme, a Kringle domain adjacent to the transmembrane domain. Dror [17] and Dnrk [18] in *Drosophila* have no Ig-like domains but cysteine-rich domains preceding the Kringle domain. A recent computer search [7,8] revealed that the C6 box and the fourth Ig-like domain of MuSK can also be viewed as a cysteine-rich domain (CRD) which is shared by genes of the frizzled family and has been shown to be necessary for Wnt ligand binding [19]. Previous cloning experiments revealed the existence of a number of alternatively spliced MuSK [2] and nsk2 isoforms [9]. One isoform in rat contained an insertion of eight amino acids instead of an alanine residue at position 454 and differences were also observed in the carboxy-terminal region of the mouse MuSK homologue nsk2. The identification of a novel splice variant lacking a potential functional binding/regulatory Ig-like domain provides a 'natural mutant' which might help in understanding the functional roles of the extracellularly exposed domains.

The Ig-like domain – as originally recognized in immunoglobulins – typically contains two conserved cysteine residues separated by about 70 amino acid residues which form intramolecular disulfide bonds to stabilize the tertiary structure of the protein. Such structural motifs are found in a variety of cell surface proteins including cell adhesion molecules as well as receptor tyrosine kinases. Genomic DNA analysis revealed that the Ig-like domains either are encoded by single exons or are located on two exons each encoding one of the two cysteine residues on 'half a domain' [20] and thus could increase the variety of alternatively spliced isoforms of proteins belonging to the immunoglobulin super gene family.

The MuSK-ΔIgIII isoform results from splicing events that eliminate two exons which each carry a 'half a domain' of IgIII. The transcripts of the two MuSK isoforms, as detected by PCR, are expressed at different levels. The shorter transcripts are about 10 times less abundant than the predominating longer transcripts and similar ratios are observed during late embryonic development (embryonic days E17, E19) and at birth (P1). Both forms are strongly reduced with further development. Denervation of the leg muscles in adult animals has been shown to increase the MuSK transcript levels by about 10-fold. There is also a significant increase for MuSK-ΔIgIII transcripts indicating that both forms are correspondingly regulated during the developmental stages analyzed and by muscle activity. This behavior is reminiscent of the changes observed for the muscle activity-sensitive transcripts of the AChR α, β, γ and δ subunits which decrease after birth and are upregulated upon denervation [21,22] and indicates that similar regulatory mechanisms could determine the expression of MuSK and AChR [14].

The availability of the IgIII-lacking variant provides a way to investigate the functional role of the various motifs of the MuSK ectodomain. Gene transfer into individual muscle fibers using both MuSK forms indicates that the IgIII domain is not required for the basic kinase activation. The kinase is activated upon overexpression of both receptor tyrosine kinase isoforms and induces AChR clusters in ectopic regions of muscle.

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